

Lim et al., Afr J Tradit Complement Altern Med., (2017) 14 (5): 113-120<https://doi.org/10.21010/ajtcam.v14i5.15>**EFFECTS OF GLYCYRRHIZIN PRE-TREATMENT ON TRANSIENT ISCHEMIC
BRAIN INJURY IN MICE****Chiyeon Lim^{1†}, Sehyun Lim^{2†}, Young-Jun Lee³, Bokcheul Kong⁴, Byoungcho Lee⁵, Chang-
Hyun Kim¹, Buyeo Kim⁶, Suin Cho^{7*}**

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Corresponding Author E-mail:**sicho@pusan.ac.kr**Article HistoryReceived:* 16, July. 2016*Revised Received:* 19, Sept. 2016*Accepted:* 22, Sept. 2016*Published Online:* 01, Oct. 2017**Abstract**

Background: Ischemia-induced brain damage is the leading cause of adult disability and the fifth leading cause of death, and thus, the development of anti-apoptotic neuro-protective therapeutic agents is viewed as an attractive developmental strategy. Glycyrrhizin is the main sweet component in licorice and has a number of pharmacological activities, which include neuro-protective, anti-fungal, and anti-cariogenic activities. This study was undertaken to investigate the effects of glycyrrhizin on ischemia-induced brain damage.

Materials and Methods: In infarct volumes and the levels of several apoptosis-related proteins, caspase-3, -8, 9, Bcl-xL, Bcl-2, and their activities in the brains of middle cerebral artery occlusion (MCAO) treated mice were measured using western blotting methods.

Results: Single pre-treatment with glycyrrhizin (10-100 mg/kg) at 2 hours before MCAO significantly reduced infarct volumes at 24h after MCAO. In addition, glycyrrhizin effectively inhibited the activations of caspase-3 and -9 and the down-regulation of Bcl-xL protein caused by MCAO.

Conclusion: The neuro-protective effect of glycyrrhizin was found to be due to its regulation of apoptosis-related proteins signals. The authors suggest glycyrrhizin be considered a potential candidate for the treatment of ischemia induced brain damage.

Keywords: Glycyrrhizin, licorice, stroke, apoptosis

Abbreviations: MCAO, middle cerebral artery occlusion; rCBF, relative cerebral blood flow; TTC, 2,3,5-Triphenyl-tetrazolium chloride.

Introduction

Glycyrrhizin (glycyrrhizic acid) is one of main constituents of *Glycyrrhiza glabra* or *G. uralensis* (licorice) roots, and has been shown to have a number of pharmacological activities, which including neuro-protective (Kim et al., 2011; 2012; Luo et al., 2013), anti-fungal (Guo, 1991), and anti-cariogenic effects (Segal et al., 1985). Isoliquiritigenin, a natural phenol isolated from licorice, has been reported to have protective effects on transient MCAO induced focal cerebral ischemia in rats (Zhan and Yang, 2006). In total, 122 compounds have been isolated from *G. uralensis* and extensively tested using various bioassay methods (Ji et al., 2016; Ota et al., 2015; Yang et al., 2015b; Zhu et al., 2016). Kumagai et al. (1957) reported glycyrrhizin competitively inhibits the metabolism of corticosteroids and that it has a corticosteroid-like effect (Kumagai et al., 1957).

Corticosteroids are often administered by clinicians, but evidence concerning their effects on acute cerebral infarction is lacking. However, corticosteroids are believed to reduce cytotoxic and vasogenic brain edema when administered to ischemic cerebral stroke (Faraji et al., 2009; Feigin et al., 2005; Sandercock and Soane, 2011).

Stroke is a leading cause of adult death and permanent disability, and ischemic stroke accounts for around 80% of strokes (Poisson et al., 2014). Furthermore, the incidence of stroke is increasing among young adults (Bejot et al., 2016).

Ischemia/reperfusion-induced stroke can be characterized by focal loss of brain blood circulation, and often leads to neuronal injury or death (Hadadha et al., 2015; Zhao et al., 2015). Kim et al. (2012) concluded that the anti-inflammatory and anti-oxidative effects of glycyrrhizin were responsible for its neuroprotective effect post-ischemia in a rat MCAO model (Kim et al., 2012). However, the effects of glycyrrhizin have not been determined in a mouse MCAO model of ischemia. Thus, in the present study, we sought to determine whether glycyrrhizin protects against ischemia/reperfusion induced brain damage and to identify the mechanism responsible in a mouse MCAO model.

Materials and Methods

Glycyrrhizin treatment

Glycyrrhizin was purchased from Sigma-Aldrich (Millipore Sigma, MO, USA), dissolved in dimethyl sulfoxide (DMSO) to concentration of 100 mg/ml, and then again diluted with tap water to concentration of 2 mg/ml.

Animal model

The animal experiment protocol was approved by the ethics committee of our institution (approval number, PNU-2016-1087). Male SPF C57BL/6 mice (Daehan Biolink, Chungbuk, Korea) (22-25 g) were housed in a humidity and temperature controlled environment under a 12h light cycle, and provided food and water *ad libitum*. For the experiment animals were randomly divided into five groups, namely, the sham control group (members of which underwent surgery but not MCAO), the non-glycyrrhizin treated MCAO group (the MCAO control group), and the 10, 30, or 100 mg/kg glycyrrhizin treated groups (the 10, 30, and 100 mg/kg groups) each group consisted of at least 15 animals.

Before MCAO, mice in glycyrrhizin treatment groups were orally administered 10, 30, or 100 mg/kg of body weight 1 hr before commencing the MCAO procedure (Fig. 1(A)). MCAO was conducted under 2% isoflurane anesthesia. During surgery, rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a heating pad, and relative cerebral blood flow (rCBF) was monitored using a laser Doppler blood flow system (moorVMS-LDF, Moor Instruments, Devon, UK). Briefly, under a stereo microscope (Nikon 745, Tokyo), the left external carotid artery and the left common carotid artery were bound with 4/0 silk sutures (Ethicon Inc., NJ, USA). The left middle cerebral artery was then occluded using a filament (11 mm length of 8/0 nylon suture with a silicon-coated tip), and after 2h of occlusion, the filament was withdrawn. The success of the procedure was confirmed by a reduction in rCBF of $>80\%$ during the 2h ischemic period, and a sharp increase in rCBF to $>90\%$ of baseline during reperfusion. Surgical procedures performed in an identical

manner in the sham group but the left middle cerebral artery was not occluded. Mice were anesthetized and sacrificed 24 hr after commencing MCAO.

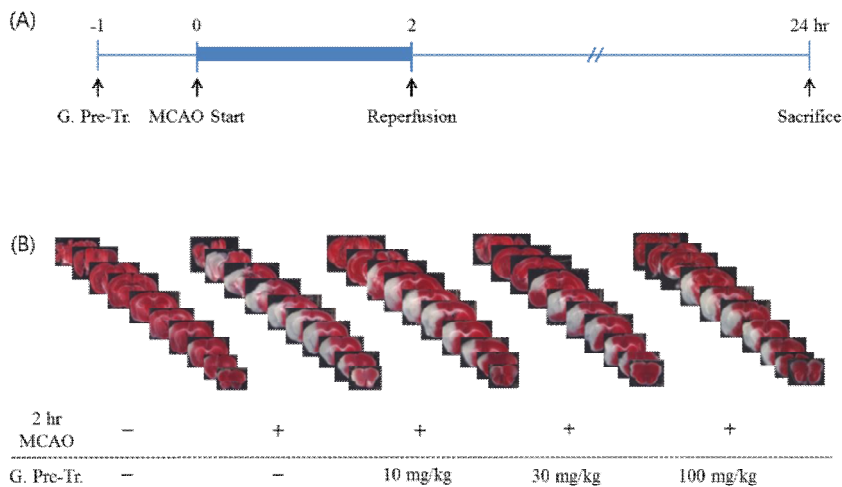


Figure 1: Design of the MCAO model and representative group images. (A) Glycyrhizin was pre-treated 1 hr before commencing MCAO, and mice were sacrificed 24 hr after commencing MCAO (24h post-MCAO). Harvested brain slices were kept in deep freezer for protein assays, or stained with TTC to measure infarct volumes. (B) Ischemic regions were identified as pale regions in coronal slices.

Measurement of infarct volumes

Brains harvested at 24h post-MCAO were immediately sectioned at 1 mm coronal sections to obtain 10 sections/brain. Sections were then incubated with 2% 2,3,5-triphenyl-tetrazolium chloride (TTC) solution for 17 min at 25°C and fixed in 10% neutral buffered formalin for more than 2 hr. Digital images were obtained using a digital camera, and infarct and relative edema volumes were calculated using Image J software (NIH, Maryland, USA).

Neurological deficit scores

Neurological deficit scores were assessed at 24 hr post-MCAO using a five-point scale. Briefly, the scores were calculated as follows: 0, no neurological deficit; 1, failure to extend right forepaw and a reduced grip; 2, spontaneous movement in all directions and circling to the right when the tail was pulled; 3, circling or walking to the right when stimulated; 4, unresponsive to stimulation or stroke-related death.

Western blot analysis

Ipsi-lateral brain tissues including hippocampus and cortex were extracted and homogenized in tissue lysis buffer. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes (Whatman, Maidstone, UK). Membranes were blocked using 5% skim milk in TBST buffer for 1 hr at room temperature and then incubated overnight at 4°C with specific antibodies for caspase-3, -8, 9, Bcl-xL, Bcl-2 (1:1000), and β -actin (1:2000) for internal control. Antibodies listed above were purchased from Cell Signaling Technology (Danvers, MA, USA). After overnight incubation, horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG, pAb (1:5000) and HRP conjugated goat anti-mouse IgG pAb (1:3000) were added for 2 hours. Membranes were then treated with ECL solution (GenDEPOT, Houston, TX, USA) and protein bands were detected using a photosensitive luminescent analyzer system (AmershamTM Imager 600, UK). Relative

protein amounts were analyzed using the Image J program (NIH, Maryland, USA) versus β -actin.

Statistical analysis

One-way ANOVA was used to determine the statistical significance of differences. Data are expressed as means \pm standard deviations (means \pm SD). SIGMAPLOT 12.0 version was used to statistical analysis, and p -values of ≤ 0.05 were considered statistically significant.

Results

Effects of glycyrrhizin on behavioral deficits and infarct volumes

At 24h post-MCAO, motor behavioral deficit scores were significantly higher in mice subjected to MCAO. Glycyrrhizin pre-treatment exhibited no significant reduction in neuronal deficit scores versus the MCAO control group (Figure 2(A)). Regions of ischemic infarction were confirmed by TTC staining, and MCAO was found to induce severe damage in ipsilateral hemispheres. However, mice pre-treated with glycyrrhizin (10, 30, or 100 mg/kg) had significantly smaller infarct lesions than MCAO controls (Figure 2(B)).

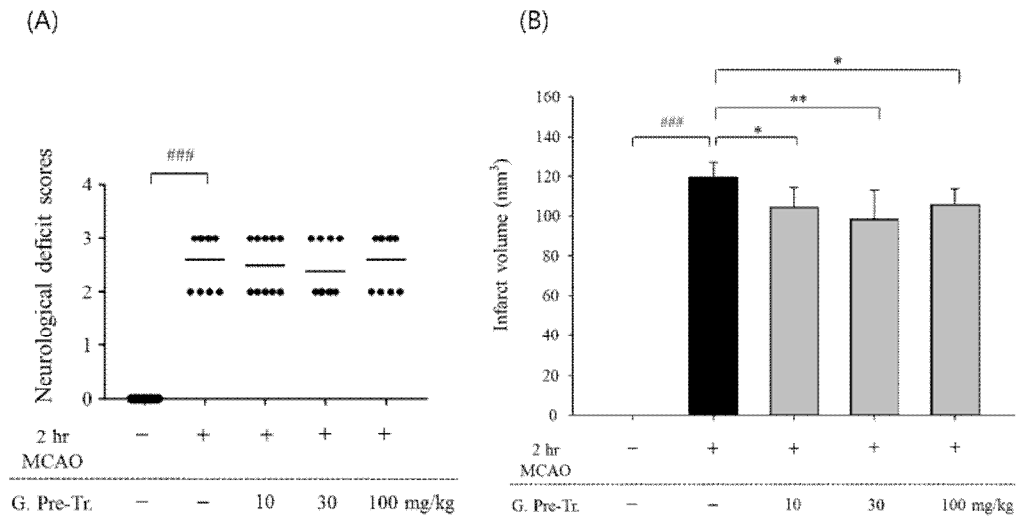


Figure 2: Effects of glycyrrhizin on neurological deficit scores and infarct volumes. Glycyrrhizin pre-treatment did not improve neuronal deficit scores (A), but significantly decreased infarct volumes at 24h post-MCAO (B). Results are presented as means \pm SDs. ### $p < 0.001$ vs. Sham controls, * $p < 0.05$, ** $p < 0.01$ vs. MCAO controls; $n = 8$ in (A), $n = 6$ in (B).

Effects of glycyrrhizin on the expressions of apoptosis related proteins

In ipsilateral hemispheres of MCAO subjected brains, western blot analysis at 24-post MCAO revealed Bcl-xL levels were significantly lower than in sham controls (Figure 3). However, mice in the 10, 30, and 100 mg/kg groups had significant higher Bcl-xL levels than mice in the sham control group (Figure 3). Glycyrrhizin pre-treatment down-regulated caspase-3 and caspase-9 in ischemic brains, but caspase-8 levels were similar in all five study groups (Figure 4).

Western blot analysis at 24h-post MCAO revealed Bcl-2 expression was significantly lower in all MCAO groups than in sham controls (Figure 5).

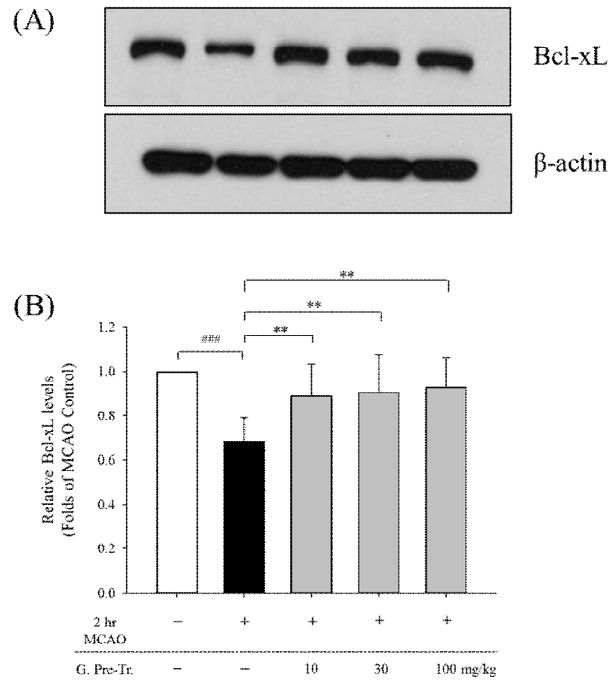


Figure 3: Effects of glycyrrhizin on Bcl-xL levels in the brains of MCAO-induced mice. Western blotting showed glycyrrhizin blocked the down-regulation of Bcl-xL by MCAO. Results are presented as means \pm SDs. ### p <0.001 vs. Sham controls, ** p <0.01 vs. MCAO controls; n =5.

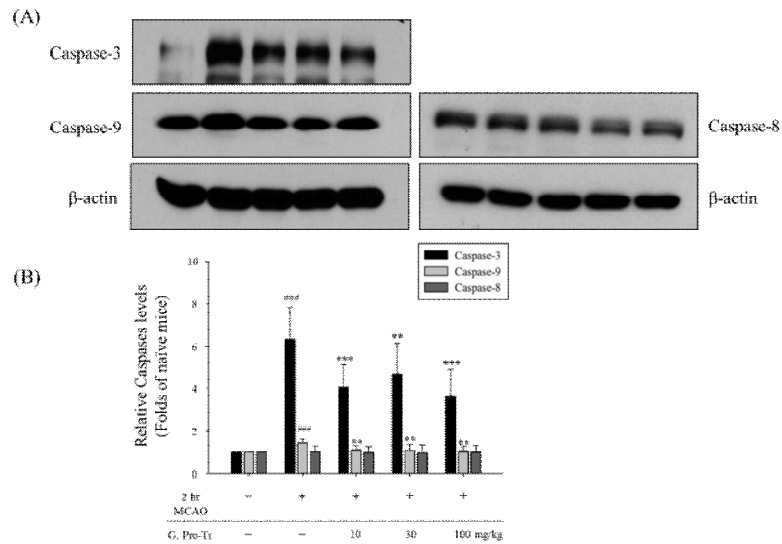


Figure 4: Effects of glycyrrhizin on brain caspase levels. Caspase-3, -8, and -9 were assessed by Western blotting. Glycyrrhizin was found to block the MCAO-induced up-regulations of caspase-3 and -9. Results are presented as means \pm SDs. ### p <0.001 vs. Sham controls, ** p <0.01, *** p <0.001 vs. MCAO controls; n =5.

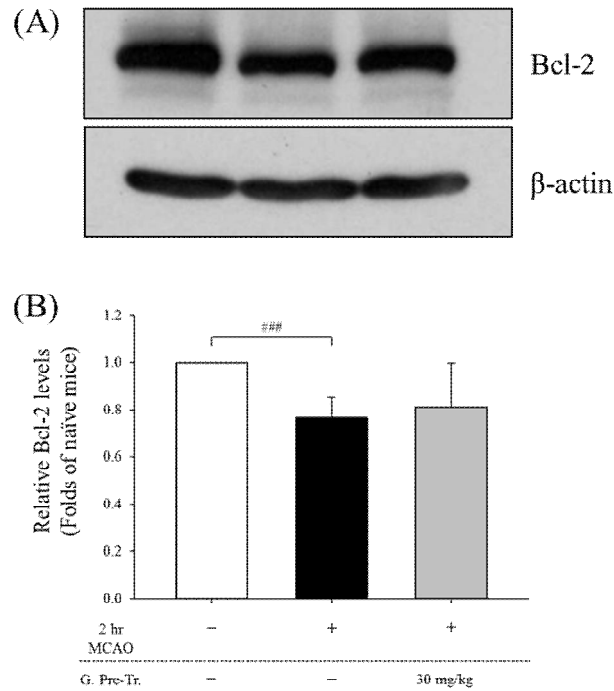


Figure 5: Effects of glycyrrhizin on brain Bcl-2 levels. Western blotting showed Bcl-2 levels were suppressed by MCAO and that glycyrrhizin had no effect on this suppression. Results are presented as means \pm SDs. ^{###} $p < 0.001$ vs. Sham controls; $n = 5$.

Discussion and Conclusion

Ischemia-induced brain damage, also called “stroke” or “brain attack”, is associated with oxidative stress and neuro-inflammation, in turn, cause diverse motor and speech disorders (Chamorro et al., 2016; Roth and Liesz, 2016). A stroke can occur at any time, and in the USA, nearly 800,000 people experience a new or recurrent stroke annually. Stroke is the leading cause of adult disability and the fifth-most leading cause of death (Bejot et al., 2016; Hachinski and Azarpazhooh, 2016; Poisson et al., 2014). The severities of motor and speech disorders depend on the extent of brain damage (Barlow, 2016), and thus, anti-apoptotic neuro-protective therapeutic agents offer an attractive developmental strategy.

In a preliminary study, we observed oral glycyrrhizin reduced brain infarct volumes in MCAO-treated mice, and in the present study, single doses of glycyrrhizin of from 10 to 100 mg/kg produced similar results (Figures 1 and 2). In order to identify beneficial effects of glycyrrhizin, we assessed the levels of several apoptosis-related proteins and their activities in ipsilateral brains.

Caspases are classified based on the roles they play during apoptosis. Caspases-8 and -9 are called “initiator caspases”, because they activate executioner caspases (caspases -3, -6, and -7), which induce the destruction of key structural proteins. Caspase-3 is responsible for DNA fragmentation and plays a critical role in apoptosis (McIlwain et al., 2015; Sun et al., 2015). Recently, several researchers have suggested ischemia/reperfusion-induced brain injury might be ameliorated by inhibiting caspase-3 signaling (Wen et al., 2016; Yang et al., 2015a), and in the present study, glycyrrhizin was found to inhibit increase in caspase-3 and -9 levels caused by MCAO-induced brain damage (Figure 4).

The Bcl-2 and caspase families play act as regulators of the apoptotic pathway, and interestingly, members of this family act as positive or negative regulators. Bcl-xL is an anti-apoptotic member of the Bcl-2 family (Dragovich et al., 1998; Wu and Tang, 2016), and in the present study, its levels, and those of Bcl-2,

were significantly lower in damaged brains. Furthermore, glycyrrhizin pretreatment effectively inhibited reduced this MCAO-induced Bcl-xL down-regulation (Figure 3, Figure 5).

Our results suggest that glycyrrhizin pretreatment decreased infarct volumes in the brains of MCAO-treated mice by regulating apoptosis-related protein signaling (Figure 6). Accordingly, our findings indicate that glycyrrhizin is a promising candidate for the prevention or treatment of ischemia-induced brain damage.

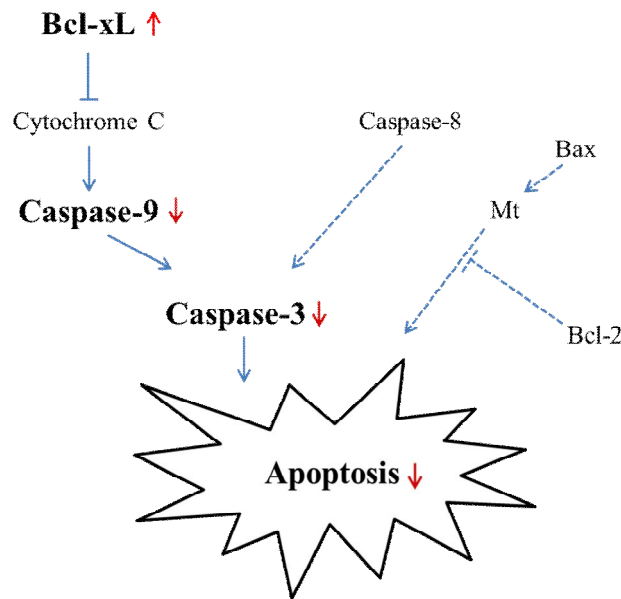


Figure 6: Schematic of the anti-apoptotic action effect of glycyrrhizin in the MCAO mouse model. Red arrows indicate predicted effects of glycyrrhizin on ischemia/reperfusion induced brain damage.

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